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VIRAL VACCINE IMMUNOGENICITY IN RELATION TO HOST CELL-MEDIATED AND HUMORAL IMMUNE RESPONSES

FINAL REPORT

by

Stanley G. Rabinowitz, M.D.

May 1976

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Immune reponses after immunization with a number of inactivated Venezuelan equine encephalomyelitis (VEE) virus vaccines were evaluated using an adoptive transfer system. Formalin-inactivated, TC-83 strain VEE virus vaccine was found to be immunogenic and highly effective in protecting recipients against challenge with virulent VEE virus. In contrast to immunization with live, TC-83 VEE virus vaccine, immunization with inactivated vaccine did not provide donors with the capacity to transfer adoptive immunity readily. Only when mice were immu-

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nized with inactivated VEE vaccine combined with adjuvants, particularly complete Freund's adjuvant or Bordetella pertussis, were donors capable of consistently transferring adoptive immunity.

Lymphoid cell responses to immunization with inactivated VEE vaccine was next assessed by monitoring the development of both donor serum neutralizing antibody as well as adoptive neutralizing antibody responses induced by spleen cell transfer. Donors immunized intraperitoneally with formalin-inactivated VEE vaccine singly or on 3 consecutive days develop early and brisk serum neutralizing antibody responses ($\geq$ 1:88 - 1:100) by 7 days within immunization. Recipients of spleen cells prepared from such mice are, however, incapable of eliciting a neutralizing antibody response ($\leq$ 1:10). Only spleen cells prepared from donors immunized with inactivated VEE vaccine combined with adjuvants, particularly complete Freund's adjuvant and Bordetella pertussis, are consistently capable of producing early and brisk serum neutralizing antibody responses in adoptively immunized recipients ($\geq$ 1:50 - 1:120 on day 4 after cell transfer). The magnitude of donor neutralizing antibody responses to inactivated VEE vaccines did not serve as a useful guide as to whether spleen cells derived from such mice could adoptively induce antibody responses in recipients.

In vitro spleen cell stimulation was also employed as a correlate of cellular immunity. Combining inactivated TC-83 vaccine with adjuvants, particularly CFA and B. pertussis, resulted in augmentation of spleen cell proliferation in response to VEE antigen.

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SUMMARY

Immune responses after immunization with a number of inactivated Venezuelan equine encephalomyelitis (VEE) virus vaccines were evaluated, using an adoptive transfer system. Formalin-inactivated, TC-83 strain VEE virus vaccine was found to be immunogenic and highly effective in protecting recipients against challenge with virulent VEE virus. In contrast to immunization with live, TC-83 VEE virus vaccine, immunization with inactivated vaccine did not provide donors with the capacity to transfer adoptive immunity readily. Only when mice were immunized with inactivated VEE vaccine combined with adjuvants, particularly complete Freund's adjuvant or Bordetella pertussis, were donors capable of consistently transferring adoptive immunity readily.

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Analysis of donor and adoptively-immunized recipient serum neutralizing antibody class kinetics revealed the following: Donor mice immunized with either live or inactivated VEE virus vaccine combined with potent adjuvants develop specific anti-VEE IgM and IgG responses as early as 7 days post-immunization. Anti-Vee IgM antibody responses comprise the majority of anti-VEE neutralizing antibody at this early time period. By 14 to 21 days post-immunization, anti-VEE IgG responses predominated. When adoptively immunized recipients were studied, the anti-VEE IgM to IgG predominance seen in donors early after administration was reversed, and for each time period studied, recipients' serum anti-VEE antibody class responses consisted principally of igG rather than IgM antibody. Since T-cells cooperation with B-cells is critical in the IgM-IgG antibody shift, these studies support the critical role T-cells exert in adoptive transfer in a murine model of experimental VEE infection. Furthermore, immunization with either live or inactivated VEE vaccine coupled to a potent adjuvant induce comparable donor and adoptively-immunized recipient anti-VEE antibody class responses.

In vitro spleen cell stimulation was also employed as a correlate of cellular immunity. Combining inactivated TC-83 vaccine with adjuvants, particularly CFA and *B. pertussis*, resulted in augmentation of spleen cell proliferation in response to VEE antigen.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animals Resources, National Academy of Sciences - National Research Council.

PROBLEM AND BACKGROUND

During the last decade a substantial amount of immunological investigation has provided new information concerning the nature of the host's immune responses to viral infection. It has become clear that in addition to production of specific neutralizing antibody capable of inactivating many viruses, an entirely separate arm of the host's immune response exists. which is termed cell-mediated immunity (CMI). Cell-mediated immunity is capable of exerting antiviral activity either by direct lymphocyte or macrophage killing of virus-infected cells in response to surface antigenic changes induced by the virus, or by elaboration of interferon or other lymphokines (soluble mediators of potent biologic activity produced by stimulated lymphocytes) (1-9). Thus any current approach to the subject of antiviral immunity must, particularly as related to anti-viral vaccines, include studies directed at investigating B-cell, T-cell and macrophage responses.

Thymus-derived lymphocytes (T-cells) are the cells which participate in graft vs. host disease, allograft rejection, delayed hypersensitivity phenomena, helper cell function in antibody synthesis and cellular immunity to microorganisms. It is probable that different T-cell subsets are responsible for various immunological functions ascribed to T-cells. On the other hand, bone-marrow derived lymphocytes (B-cells) ultimately differentiate into cells responsible for the production of antibody. Macrophages are mobile or fixed tissue phagocytes possessing potent antimicrobial capability and, like B-cells, are derived from bone-marrow precursors.

In our previous papers (10,11) on host defenses during primary VEE virus infection in mice, it was reported that administration of both immune serum or spleen cells to nonimmune adoptive hosts conferred substantial protection against lethal infection with a virulent strain of VEE virus. Evidence was presented that thymus-dependent lymphocytes in the spleen cell population were responsible for this adoptive transfer of immunity. In addition, utilizing in vitro lymphocyte stimulation studies, spleen cell populations obtained from immune mice were shown to contain antigen-reactive cells which synthesized DNA specifically when exposed to homologous viral antigen in vitro. This antigen-induced proliferation of thymus dependent lymphocytes was shown to correlate with the protective capcity of the cell population and with the state of immune response following administration of VEE virus. Furthermore, we have extended these observations by demonstrating in vitro that immune T-cells from the spleens of VEE immunized mice activate normal macrophages co-cultivated with virus-infected feeder cells and inhibit VEE viral growth (12). Such studies have documented that potent anti-VEE activity resides not only in specific neutralizing antibody but also in CMI. These studies, however, were carried out almost exclusively utilizing the live attenuated, TC-83 strain VEE virus. In some experiments immune spleen cells were employed following immunization of donors with a formalin-inactivated, TC-83 strain vaccine. In contrast to results obtained following immunization with attenuated

virus vaccine, spleen cells harvested 8-14 days following inactivated vaccine administration were neither able to adoptively transfer protection to recipient mice nor to exhibit any antiviral effect when co-cultivated with VEE-infected cells in vitro (12). This occurred in spite of the fact that donor mice developed serum neutralizing antibody responses and were, moreover, fully resistant to lethal challenge with VEE virus. Recent studies (13,14) have extended these observations further. These studies have suggested that for VEE vaccines, as for other protein antigens (15), the nature of the immune response induced after immunization depends on several factors including: (1) physio-chemical state of the antigen, i.e., live vs. inactivated; (2) the dose of antigen used; (3) number of boosts of antigen given; (4) whether adjuvants are usedin the immunization schedule; and (5) the nature of the adjuvant itself. Furthermore, immunization with inactivated VEE vaccine alone appears insufficient to produce intense and/or durable lymphoid cell responses.

RESULTS

I. Effect of different immunization regimens on capability of donor spleen cells to transfer adoptive immunity

Results in this section have been published in the July, 1976 issue of the Journal of Infectious Diseases (13). A brief summary of the findings follow. Cellular immune responses after immunization with a number of inactivated VEE virus vaccines were evaluated using an adoptive transfer system. Formalin-inactivated, TC-83 strain VEE virus vaccine was found to be immunogenic and highly effective in protecting recipients against challenge with virulent VEE virus. In contrast to immunization with live, TC-83 VEE virus vaccine, however, immunization with inactivated VEE vaccine did not provide donors with the capacity to transfer adoptive immunity readily. Only when mice were immunized with inactivated VEE vaccine combined with adjuvants, particularly complete Freund's adjuvant or Bordetella pertussis, were donors capable of consistently transferring adoptive immunity. Total dose of inactivated VEE vaccine did not appear to influence the capacity to transfer adoptive immunity. On the other hand, boosting weekly with VEE vaccine and/or administration of vaccine with specific adjuvants did markedly influence donor immune responses.

II. Effect of different immunization regimens on kinetics of serum neutralizing antibody responses in donor and cell transfer recipients

Results in this section have been published in the July, 1976 issue of the Journal of Infectious Diseases (14). A brief summary of the findings follow. Lymphoid cell responses to immunization with various formalin-inactivated VEE virus vaccines were monitored by assessing the development of

both donor serum neutralizing antibody as well as adoptive neutralizing antibody responses induced by spleen cell transfer. Donors immunized intraperitoneally with formalin-inactivated VEE vaccine singly or on 3 consecutive days develop early serum neturalizing antibody responses ($\geq$ 1:88 - 1:100) by 7 days after immunization. Recipients of spleen cells prepared from such mice are, however, incapable of eliciting a neutralizing antibody response ($\leq$ 1:10). Only spleen cells prepared from donors immunized with inactivated VEE vaccine combined with adjuvants, particularly complete Freund's adjuvants and Bordetella pertussis, are consistently capable of producing early high titer serum neutralizing antibody responses in adoptively immunized recipients ($\geq$ 1:50 - 1:120 on day 4). The magnitude of donor neutralizing antibody responses to inactivated VEE vaccines did not serve as a useful guide to whether spleen cells derived from such mice could adoptively induce antibody responses in recipients. Treatment of immune spleen cells with anti-thymocyte serum, but not anti-mouse γ-globulin, or normal rabbit sera abrogated the capacity of such cells to adoptively transfer an antibody response.

III. Effect of different immunization regimens on in vitro spleen cell stimulation to VEE antigen.

Spleen cell stimulation in vitro in response to inactivated VEE antigen was assessed as a correlate of cellular immunity. Spleen cells harvested 7-28 days after immunization with inactivated VEE vaccine demonstrated minimal stimulation at 7, 14, and 21 days after vaccination (Table 1). Similarly, mice immunized with inactivated vaccine once daily for 3 consecutive days also failed to demonstrate a substantial proliferative response to VEE antigen in vitro (Table 1). In contrast, mice immunized once weekly with inactivated VEE vaccine demonstrated a substantial proliferative response at 7, but not 14 or 21 days after vaccination (Table 1). Similarly, substantial early (6-13 day) spleen cell proliferative responses were evident in mice immunized with inactivated VEE vaccine combined with adjuvant (Table 2). Clearly, however, spleen cell stimulation was most pronounced in the groups receiving inactivated VEE vaccine with either CFA or B. pertussis.

IV. Effect of different immunization regimens on kinetics of immunoglobin antibody classes appearing in donor and cell transfer recipients.

To prepare IgM fractions of mouse sera following immunization with inactivated VEE vaccine, exclusion chromatography employing G-200 Sephadex was employed. Pharmacia columns (1.5 cm x 90 cm) were packed with G-200 Sephadex, appropriately swelled by boiling for 5 hours in a solution consisting of 0.1M Tris-HCl and 0.2M NaCl. The column was packed under gravity and equilibrated for 18 hours with buffer (0.1M Tris-HCl, 0.2M NaCl). One to 2.0 ml of serum was then applied to the top of the Sephadex and the flow rate adjusted to 2 ml/cm^2/hour by use of a peri-

staltic pump. Two ml fractions were collected by an automatic fraction collector and scanned spectrophotometrically at 280 nm to determine the protein distribution in the effeulent fractions.

Donor mice immunized with either live or inactivated VEE virus vaccine combined with potent adjuvants develop specific anti-VEE IgM and IgG responses as early as 7 days post-immunization. At this early time period, anti-VEE IgM antibody responses predominate. By 14-21 days post-immunization, specific anti-VEE IgG responses predominate. When adoptively immunized recipients were studied the anti-VEE IgM to IgG predominance seen in donors early after immunization was reversed; and for each time period studied, recipients' serum anti-VEE antibody class responses consisted principally of IgG rather than IgM antibody.

Our previous studies have amply documented that following immunization with inactivated VEE virus vaccines combined with potent adjuvants, donor spleen cells possess the capacity to adoptively-transfer protection against VEE virus challenge. Furthermore, this protection correlated with the capacity of the adoptively transferred spleen cells to induce early serum neutralizing antibody response in syngeneic recipients. Our current studies extend these observations and suggest that strong specific anti-VEE IgG serum antibody responses are produced in adoptively immunized recipients. In fact, major early anti-VEE antibody IgG responses in recipients appear to occur at a time when donor anti-VEE antibody is principally of the IgM class. Clearly, however, since the tranferred cell preparations circulate in recipients for some time prior to testing, care must be exercised in judging the magnitude and character of the antibody response seen in recipients and donors.

DISCUSSION

Inactivated VEE vaccines are immunogenic and highly effective in protecting recipients against challenge with VEE virus. The duration of this protection, although not studied, is at least several weeks. In contrast to immunization with live, TC-83, VEE vaccine, immunization with inactivated VEE does not provide donors with the capacity to transfer adoptive immunity readily. Only when mice were immunized with inactivated vaccine combined with adjuvants were donors capable of consistently transferring adoptive immunity. Total dose of VEE antigen, within the limits of the study, did not appear to influence the capacity to transfer adoptive immunity. On the other hand, duration of antigen exposure and/or administration of antigen with adjuvant did markedly influence donor immune responses.

In studying the kinetics of serum neutralizing antibody development following immunization with inactivated VEE vaccines, several points emerged. First, the magnitude of donor antibody response following immunization bore no relationship to the capacity to transfer adoptive immunity.

For example, serum neutralizing antibody responses in mice immunized with I-TC-83 with B. pertussis were not significantly different than antibody responses in mice immunized with I-TC-83 x 3. Yet mice given I-TC-83 with B. pertussis consistently were able to transfer adoptive immunity. In addition, donor serum antibody responses 14 and 21 days after administration of I-TC-83 and CFA were significantly different, yet both groups of donors appeared to possess equal capacity to transfer adoptive immunity. It does not appear, therefore, that the height of donor serum antibody response at the time cell transfer occurs is a useful index of the competence of transferred cells to induce immunity.

Second, in studying antibody responses in adoptively-immunized recipients, correlation existed between the development of early ($\leq$ 4 days) serum neutralizing antibody titer equal to or greater than 1:40 and protection against VEE virus challenge. Thus, both groups of mice receiving spleen cells following immunization with I-TC-83 and adjuvant were able to produce early, high titer serum neutralizing antibody (1:40 - 1:120) and to resist VEE virus challenge. That is not to say, however, that neutralizing antibody is solely responsible for adoptive immunity, but it suggests that antibody plays an important role in protection. Of some interest in this regard are studies with mice immunized weekly with I-TC-83. Here, in spite of the appearance of neutralizing antibody titers in recipients comparable to those of mice immunized with vaccine and adjuvant, only incomplete protection against VEE virus challenge developed. Furthermore, a powerful argument against antibody being solely responsible for protection comes from studies with mice immunized with I-TC-83 + CFA (s.c.). Recipient mice receiving spleen cells 7 days after donor immunization were significantly protected against virulent VEE challenge and yet no serum neutralizing antibody response could be detected in this group. Thus, while adjuvant may potentiate humoral antibody responses, it may also importantly impinge on T-cells necessary for the adoptive transfer of CMI.

Since we, as well as many others (9-12), have relied on in vitro lymphocyte assays as correlates of CMI, studies were undertaken after inactivated VEE vaccine administration, investigating whether lymphocyte stimulation occurred in response to VEE antigen. It appeared that following immunization with I-TC-83 given once or on 3 consecutive days, no significant lymphocyte stimulation was detectable (Table 1). Thus, I-TC-83 does not seem to induce a CMI response in the host. All other immunized groups, however, showed significant lymphocyte stimulation in response to VEE antigen (Tables 1 and 2). It appears, however, that lymphocyte stimulation specifically in response to antigen can reflect either T-cell and/or B-cell proliferation (11,12). For example, we have previously shown that spleen cells harvested 8 days after mice were immunized with live VEE virus vaccine and reimmunized 5 weeks later, proliferated in vitro in response to VEE antigen, but that only B-cells participated in this response (11). Thus, it is possible that stimulation reflects B-cell expansion. Nevertheless, correlation appeared good be-

tween results of lymphocyte stimulation and the capacity to transfer adoptive immunity. It thus appears clear that lymphocyte stumulation in response to vaccine serves as another marker of intense lymphoid cell response to immunization. Further work is needed in this area.

Finally, it is worthwhile considering the subject of adjuvanticity in regard to host immune responses to VEE vaccines. Adjuvant action or adjuvanticity can operationally be considered to refer to any substance which accelerates and/or enhances the immune responses engendered by antigen (22). It seems clear that no single mechanism can be invoked in defining the immunopotention induced by adjuvants. Some such as B. pertussis may impinge directly on T-cells (23), others may act on macrophages and T-cells (24), others predominantly on certain T-cell subsets rather than all T-cells (22). One explanation of the adoptive immunity induced by adjuvant and inactivated VEE vaccine is that adjuvant acts to enhance T-helper cell function. The enhancement of T-helper cell function results in a marked expansion of B-cell clones committed to anti-VEE antibody production. Adoptive transfer is successful in these circumstances because the cell preparation transferred quantitatively has much larger numbers of sensitized B-cells present. The difficulty with accepting this interpretation entirely is that neutralizing antibody responses among mice immunized with I-TC-83 and B. pertussis were not augmented in comparison to other immunized groups. If immunization with VEE vaccine and B. pertussis simply augments T-helper cell function then donor antibody responses should be augmented as seen in the I-TC-83-CFA group. Of even more importance is the absence of complete correlation between protection induced by cell transfer and antibody production in recipients. Thus, appearance of serum neutralizing antibody in recipients may be associated with cell transfer without being solely responsible for protection. Rather than explaining adjuvant enhancement of host immune responses to I-TC-83 vaccine simply in terms of augmentation of T-helper cell function, it is entirely possible that adjuvant also augments a T-cell subset responsible for cellular immunity to VEE virus. Successful transfer of immunity under these circumstances could result from both acceleration of neutralizing antibody responses and augmentation of cellular immunity.

These studies suggest that one of the significant differences between immunization with live VEE vaccine and inactivated VEE vaccine lies in the capacity of attenuated virus to interact with both T-cells and B-cells. Manipulation of inactivated VEE vaccine by combining it with adjuvant or by extending the duration of antigen stimulation through weekly immunization tend to produce host immune responses comparable to those seen with live VEE vaccine. The implication of this for vaccine immunotherapy needs further study.

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Table 1

In vitro reactivity of immune mouse spleen cells[a] to VEE viral antigen harvested at various times following immunization with inactivated VEE vaccine once, or on 3 consecutive days or once weekly for 3 weeks.

Immunizing Vaccine	Day of Cell Harvest	Lymphocyte incorporation of tritiated thymidine (Δ mean CPM)[b]		
		viral antigen dilution (ul/culture)		
		5	0.5	0.05
None		1483 ± 284	467 ± 70	543 ± 42
I-TC-83 x 1[c]	7	3227 ± 169	794 ± 49	544 ± 68
	14	2239 ± 193	962 ± 29	1091 ± 87
	21	3369 ± 206	2448 ± 103	-
	28	333 ± 18	366 ± 45	-
I-TC-83 x 3[d]	6	1934 ± 157	238 ± 326	-
	13	1608 ± 160	951 ± 39	-
	20	1330 ± 163	1021 ± 99	-
	27	2533 ± 306	3179 ± 54	-
I-TC-83 x 3 wk	7	7036 ± 624	1344 ± 126	-
	14	293 ± 25	263 ± 18	-
	21	772 ± 61	436 ± 41	-

[a] 2×10^6 spleen cells/0.5 ml/culture, incubated for a total of 48 hr with tritiated thymidine present for the last 24 hr of culture.

[b] *In vitro* lymphocyte reactivity expressed as the difference in mean counts per minute ± S.E.M. between antigen-stimulated lymphocyte cultures and the counts per minute in control non-antigen-treated lymphocyte cultures per given viral antigen dilution added. Antigen was always added in 0.05 ml volumes.

[c] I-TC-83 virus (0.3 ml) administered intraperitoneally.

[d] 3 daily injections of 0.3 ml of I-TC-83 vaccine administered intraperitoneally.

Table 2

In vitro reactivity to VEE viral antigen of immune mouse spleen cells harvested at various times following immunization with inactivated VEE vaccine given with incomplete Freund's adjuvant, complete Freund's adjuvant or *B. pertussis*[a]

Immunizing Vaccine	Day of Cell Harvest	Lymphocyte incorporation of tritiated thymidine (Δ mean CPM)[b] viral antigen dilution (ul/culture)		
		5	0.5	0.05
I-TC-83 x 3 + IFA	6	5778 ± 823	1736 ± 820	-
	13	2543 ± 164	1242 ± 36	-
	20	1735 ± 121	1613 ± 39	-
	27	669 ± 18	660 ± 24	784 ± 32
I-TC-83 x 3 + CFA	6	3075 ± 287	1422 ± 106	963 ± 46
	8	8863 ± 783	7421 ± 1036	7786 ± 964
	13	4228 ± 56	1878 ± 312	-
	20	943 ± 92	638 ± 62	-
I-TC-83 x 3 + *B. pertussis*	6	6215 ± 600	2815 ± 207	2437 ± 186
	9	6732 ± 1403	2933 ± 86	2637 ± 284
	13	1895 ± 64	1323 ± 98	-
	20	1735 ± 162	1613 ± 192	-

[a] 2×10^6 spleen cells/0.5 ml/culture, incubated for a total of 48 hr with tritiated thymidine present for the last 24 hr of culture.

[b] *In vitro* lymphocyte reactivity expressed as the difference in mean counts per minute ± S.E.M. between antigen-stimulated lymphocyte cultures and the counts per minute in control non-antigen-treated lymphocyte cultures per given viral antigen dilution added. Antigen was always added in 0.05 ml volumes.

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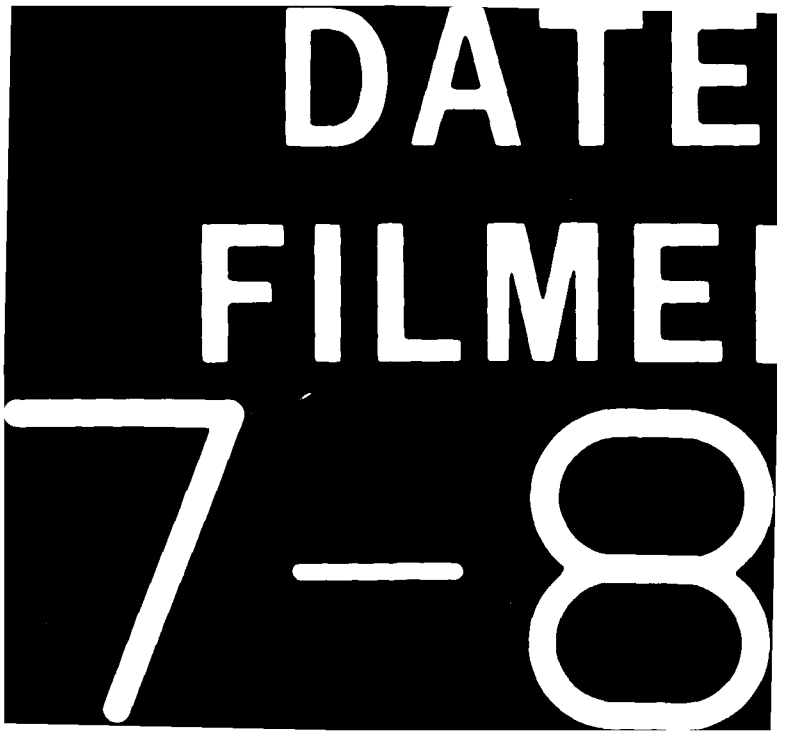
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